Expression of LGR5, an Intestinal Stem Cell Marker, During Each Stage of Colorectal Tumorigenesis

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Abstract. *Background: The Wnt target LGR5 has been recently identified as a murine intestinal stem cell marker. Its role during each stage of human colorectal tumorigenesis remains to be determined. Patients and Methods: LGR5 expression was investigated by immunohistochemical analysis in 17 low-grade and 13 high-grade intraepithelial neoplasias and 30 adenocarcinomas. Results: The number of LGR5-positive cells increased tumor, with clustering of the cells to form patches. An apparent difference in their distribution among the tumorigenesis stages was observed. LGR5 expression in luminal surface showed a negative association with the progressive grade of tumors, while that in lower crypt exhibited a positive association with grade. In adenocarcinomas, LGR5 expression in luminal surface was negatively associated with pStage, while it was almost invariably high in lower crypt during advanced pStage disease. Conclusion: These results suggest that the shifts in the distribution of LGR5-positive cells towards the lower crypt play a role in the development and progression of colorectal cancer.*

Colorectal cancer is the final stage of a step-wise process that begins with normal tissue, changes to adenoma, followed by the final stage of adenocarcinoma. This process is known as the adenoma-carcinoma sequence, and has been shown to occur in conjunction with the stepwise accumulation of distinct genetic alterations that confer a growth advantage and ultimately contribute to the progression of the lesion *via* a clonal expansion of the cells (1-3). Although it is believed that the stem cells are an ideal therapeutic target of these carcinogenic processes, due to their longevity and self-renewing properties which allow them to accumulate mutations, the lack of reliable markers for native stem cells has made it difficult to positively identify these carcinogenic target cells.

Most sporadic colorectal cancers are initiated by activation mutations of the *APC* or β-catenin gene in the Wnt pathway, which results in nuclear β-catenin accumulation and constitutive transcriptional activation by the β-catenin/T-cell factor TCF4 complex (4-6). Recently, Barker *et al.* demonstrated that leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), an orphan G-protein coupled receptor and *Wnt* target gene, are expressed exclusively in the columnar cells at the base of the crypt of the small intestine and colon in *Lgr5*-LacZ and -GFP knock-in mice (7). Lineage-tracing in *Lgr5*-EGFP-IRES-creER^{T2}/Rosa26-LacZ mice demonstrated that the Lgr5-positive crypt base columnar cells generate all epithelial lineages over a 60-day period, suggesting that they represent the stem cells of the small intestine and colon (7). Furthermore, deletion of *Apc* in the Lgr5-positive stem cells of *Lgr5*-EGFP-IRES-creER^{T2}/*Apc*$^{flox/flox}$ mice results in progressively growing adenomas, while transformed Lgr5-positive cells remain located at the bottom of the crypts, which suggests that mutated Lgr5-positive intestinal stem cells are the relevant source of colorectal neoplasia (8).

The distribution of LGR5-positive cells within the stem-cell-derived tumor is consistent with the cancer stem cell hypothesis that states that a small reservoir of self-sustaining cells is exclusively able to self-renew and maintain the tumor (9, 10). Cancer stem cells were first identified in acute myeloid leukemia (11), and more recently in solid tumors (12-16), where it was demonstrated that only a few cells are required to initiate a new tumor. These cells may be also refractory to current therapies and may be the only population of cells that are able to metastasize (9, 10, 17). Although the carcinogenic potential of these LGR5-positive tumor cells has yet to be demonstrated, LGR5 appears to be a relevant candidate marker

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of colorectal cancer stem cells. Recently, overexpression of LGR5 has been shown to occur in human colorectal adenomas and cancers (18-21), as well as in other solid tumors (19, 22, 23). However, the precise roles of LGR5 remain to be determined in each stage of the human adenoma-carcinoma sequence. The present study analyzed expression of the putative cancer stem cell marker, LGR5, in sporadic colorectal adenomas and in carcinomas by immunohistochemical analysis. The relationship between this data and the Wnt/β-catenin/TCF4 pathways was also investigated.

Patients and Methods

Tumor specimens. Primary tumor specimens from 60 colorectal tumors were consecutively obtained either by surgery or endoscopy from patients at the Hokkaido University Hospital between 2000 and 2003. Informed consent was obtained from all patients for the use of their resected tumor specimens. This study was approved by the Medical Ethics Committee of the Hokkaido University Hospital. The colorectal tumor group consisted of 32 men and 28 women, with a mean age at diagnosis of 65.7 years (range 33-86 years). The tumor classifications were determined according to the guidelines of the International Agency for Research on Cancer (IARC) and World Health Organization (WHO) (24). Tumor specimens were histopathologically diagnosed as low grade intraepithelial neoplasia (LGIN) (n=17), high-grade intraepithelial neoplasia (HGIN) (n=13) and adenocarcinoma (n=30). The pTNM classifications were determined according to the guidelines of the International Union Against Cancer (UICC) (25). Adenocarcinoma represented 9 pT_1, 2 pT_2, 16 pT_3 and 3 pT_4 stage tumors, and 18 pN_0, 5 pN_1, 5 pN_2 and 2 pN_3 stage tumors.

Antibodies. The primary antibodies used for immunohistochemistry analyses included a rabbit polyclonal antibody to LGR5 (LS-A1232; *N* human terminal extracellular domain; MBL International Co, Woburn) (18, 19), and a mouse anti-c-MYC monoclonal antibody (9E-10, sc-40; Santa Cruz Biotechnology, Heidelberg, Germany) (26, 27). The primary antibodies for TCF4 and β-catenin were used as previously described (28).

Immunohistochemical analysis. LGR5 and c-MYC expression was analyzed by immunohistochemistry. The avidin-biotin-peroxidase complex (ABC) method was used on 4 μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization. Briefly, deparaffinized tissue sections were microwaved in 0.01 M sodium citrate (pH 6.0) to retrieve the antigenicity for 25 min for LGR5 and c-MYC. The slides were allowed to cool for an additional 20 min in citrate buffer. The sections were then incubated with 3% (w/v) H_2O_2 in methanol to inhibit endogenous peroxidase activity, followed by incubation with universal protein block (Abcam ab64226, Tokyo, Japan) for LGR5 or with normal horse serum (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for c-MYC for 30 min at room temperature to block the nonspecific antibody binding sites. The sections were consecutively reacted with a rabbit polyclonal antibody against LGR5 (1:400 dilution) or with a mouse monoclonal antibody against c-MYC (1:500) at 4˚C overnight. After washing, biotinylated goat anti-rabbit IgG antibody (Vectastain Elite ABC kit) for LGR5 or biotinylated horse anti-mouse IgG antibody (Vectastain Elite ABC kit) for c-Myc, were applied for 30 min. After a subsequent washing, avidin-biotin-peroxidase complex (Vectastain Elite ABC kit) was applied for 30 min followed by peroxidase detection with a mixture of 3,3'-diaminobenzidine (DAB; Vector Laboratories; Vector Laboratories, Burlingame, CA, USA). To determine specificity of immunostaining, serial sections were similarly processed except that the primary antibodies were omitted. Sections were counterstained with hematoxylin.

Immunohistochemical evaluation. LGR5 expression was considered to be positive if membranous and/or cytoplasmic staining was present. Because LGR5 -positive cells in most tumors showed similar staining intensity, the staining rate of the positive cells but not the staining intensity was evaluated. c-MYC expression was considered to be positive if there was positive nuclear staining. c-MYC expression was evaluated as the immunohistochemistry (IHC) score, where the IHC score=(percentage of positive cells [percentage score])×(staining intensity [scored as 0 to 3]) (28). TCF4 and β-catenin were evaluated exactly the same way as for the IHC score in a previous study (28). Using the median values as the cutoff level for the staining rates or IHC scores, results were divided into a high and low group. Using a BX 40 microscope (Olympus, Tokyo, Japan), the immunohistochemical evaluations were reproducibly obtained by one investigator (K.T.) who was blinded to the status of the other immunohistological and clinical data and who assessed all evaluations twice.

Microsatellite instability analysis. Formalin-fixed paraffin-embedded tissues were evaluated by two investigators, with the areas of the slide representing the 'tumor' (highest numbers of cancer cells present) and the 'normal' tissue (no malignant tissue found) identified by hematoxylin and eosin-stained slides. DNA was extracted from macrodissected tumors using MagneSil Genomic, Fixed Tissue System (Promega, Madison, USA). The Microsatellite Instability (MSI) Analysis System consisted of five nearly monomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) that were used for MSI determination along with two polymorphic pentanucleotide markers (Penta C and Penta D) that were used for sample identification. MSI analysis was performed according to the manufacturer's directions (Promega, MSI Analysis System, ver. 1.1). Products were analyzed by capillary electrophoresis using an ABI 310 Genetic Analyzer (Applied Biosystems, California, USA). Data were separated into groups with microsatellite instability at ≥3 mononucleotide loci classified as MSI-high (MSI-H), instability at a single mononucleotide locus classified as MSI-low, and no instability at any of the loci tested designated as microsatellite stable (MSS) (29).

Statistical analyses. The associations between Lgr5 expression and categorical variables were analyzed by a χ^2 test or Fisher's exact test, as appropriate. The level of significance was set at $p<0.05$.

Results

To elucidate the roles of LGR5 during each stage of human colorectal tumorigenesis, 17 LGINs, 13 HGINs and 30 adenocarcinomas were immunohistochemically analyzed. Human skin specimens were used as positive tissue controls, as LGR5 has been shown to be expressed in the stem cell

Table I. *Relationship between LGR5 expression and clinicopathological characteristics in 60 resected human colorectal tumors.*

Characteristic		LGR5 expression		
		Low	High	*p*-Value*
Gender	Male	10	22	
	Female	12	16	0.4
Tumor grade	LGIN	7	10	
	HGIN	5	8	
	Adenocarcinoma	10	20	0.9
Adenocarcinoma				
pT	T1-2	3	8	
	T3-4	7	12	0.7
pN	N0	6	12	
	N1-3	4	8	1.0
pStage	Stage I-II	6	11	
	Stage III-IV	4	9	1.0

*χ^2 test or Fisher's exact probability test as appropriate.

population of hair follicles, such as the cells in the lower outer root sheath of anagen hair follicles in LGR5-GFP knock-in mice (30, 31). Expression of LGR5 was detected in the plasma membrane and cytoplasm of the cells in the lower outer root sheath of anagen hair follicles (Figure 1A). No immunostaining was detected in the serial sections when the primary antibodies were omitted (data not shown).

In healthy colorectal epithelia that was located adjacent to tumors, expression of LGR5 was only sporadically detected in the crypt bases with membranous and cytoplasmic localization (Figure 1B). The rare LGR5-positive cells in the normal colorectal crypts were consistent with data from previous studies (18, 20, 21), which accounted for less than 1% of entire population of epithelial cells. Scattered immunoreactive cells in the lamina propria were also noted in a previous study and led to speculation that they may represent hematopoietic cells, as bone marrow has been shown to express LGR5 mRNA in a tissue mRNA expression study (18, 32).

In contrast to normal epithelia, 87% of colorectal tumors had LGR5-positive cells with membranous and cytoplasmic localization of at least 1% in whole tumor cells. This suggests there might be frequent increases in LGR5-positive cells in colorectal tumors. The LGR5-positive cells were commonly clustered and formed patches with similar intensities (Figures 1C, D, E and F) and were not restricted to within the crypt base. Since the median rate of LGR5-positive cells evaluated in the whole tumor area was 5%, a cutoff level of 5% was used to separate the tumors into a high and a low LGR5 expression group. There was no relationship between the LGR5 expression in the whole tumor area and either the gender or tumor grade (Table I). In addition, LGR5 expression was not associated with pT, pN or any of the stages of the adenocarcinomas.

Although expression of LGR5 in the whole tumor area was similar among the different stages of tumorigenesis, there was an apparent difference in the distribution of the LGR5-positive cells observed among the stages. LGR5 expression was evaluated in each tumor area of the luminal surface, upper crypt and lower crypt for each of the different tumor grades (Figure 2). LGR5 expression in the luminal surface showed a significantly negative association with the progressive grade of tumors (p=0.0004), while there was a significantly positive association in the lower crypt (p=0.0001) (Table II). In adenocarcinoma, LGR5 expression in the luminal surface was negatively associated with pStage (p=0.02), while it was almost invariably high in the lower crypt with advanced pStage disease (Table II).

The relationships between Lgr5 and the canonical Wnt pathway of TCF4, β-catenin and c-MYC were investigated. TCF4 and β-catenin immunostaining has been previously examined using serial sections of these colorectal tumors in conjunction with evaluation of the IHC scores (28). c-MYC expression in the same tumors was also analyzed and, similar to previous reports, it was demonstrated that the c-MYC expression was detected in the tumor nucleus (26, 27), with a median IHC score of 110. Based on the cutoff level of the median IHC score, the expressions of TCF4, β-catenin and c-MYC were divided into high- and low-expression groups. While LGR5 expression was not significantly associated with the expression of TCF4, β-catenin or c-MYC, there was a slight trend toward a positive relationship between LGR5 and TCF4 (p=0.1) and β-catenin (p=0.2) (Table III). Representative immunostaining patterns for LGR5 and the other Wnt target gene proteins are shown in Figure 3. Although LGR5-positive cells were typically clustered in a patchy distribution, TCF4, β-catenin and c-MYC were broadly expressed beyond the LGR5-positive areas (Figure 3).

Because MSI-H cancer has distinctive clinicopathological and molecular features, the present study analyzed MSI of colorectal tumors. MSI-H was only observed in two adenocarcinomas, which had LGR5 staining rates of 5% and 10% (data not shown). Therefore, the majority of the results in the present study were based on sporadic tumors that were not MSI-H and thus, the definitive roles of LGR5 in MSI-H tumors remain to be determined.

Discussion

In colorectal tumors, it has been shown that there is an increase of the putative stem cells with LGR5 expression (18-21). Using immunohistochemical analysis of the colorectal tumors during each stage of tumorigenesis, this study demonstrated significant negative and positive correlations between the LGR5 expression and the progressive grades of tumors in the luminal surface and in the lower crypt, respectively. These results suggest that

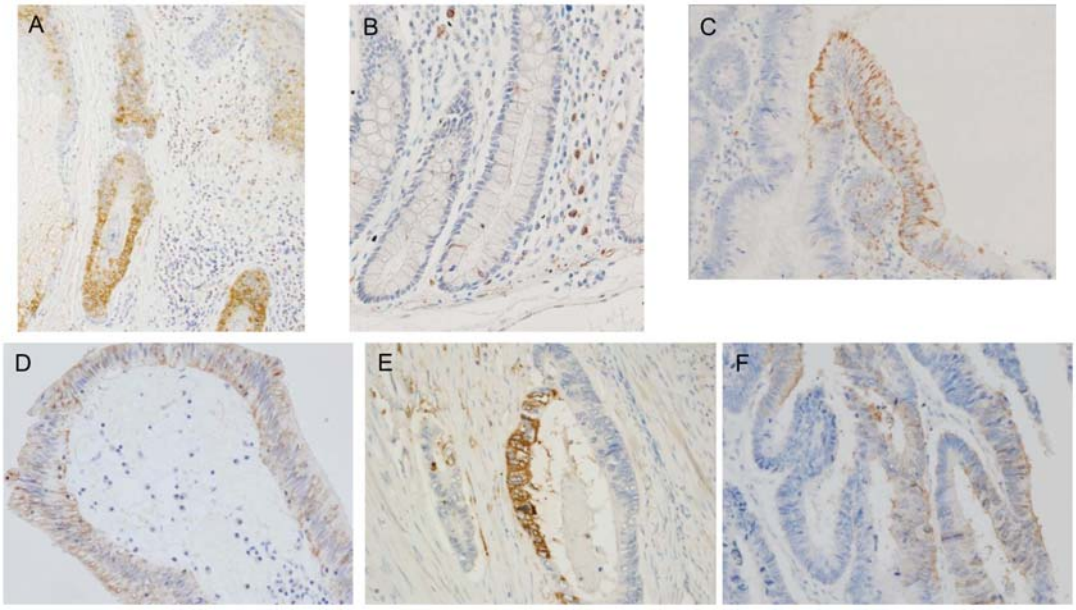

Figure 1. *Representative immunohistochemical staining patterns for LGR5 in (A) hair follicle in human skin (positive contorol) (×200), (B) crypt in normal adjacent colorectal epithelia (×200), (C) low-grade intraepithelial neoplasia (LGIN) (×400), (D) high-grade intraepithelial neoplasia (HGIN) (×400) and (E, F) adenocarcinomas (×400).*

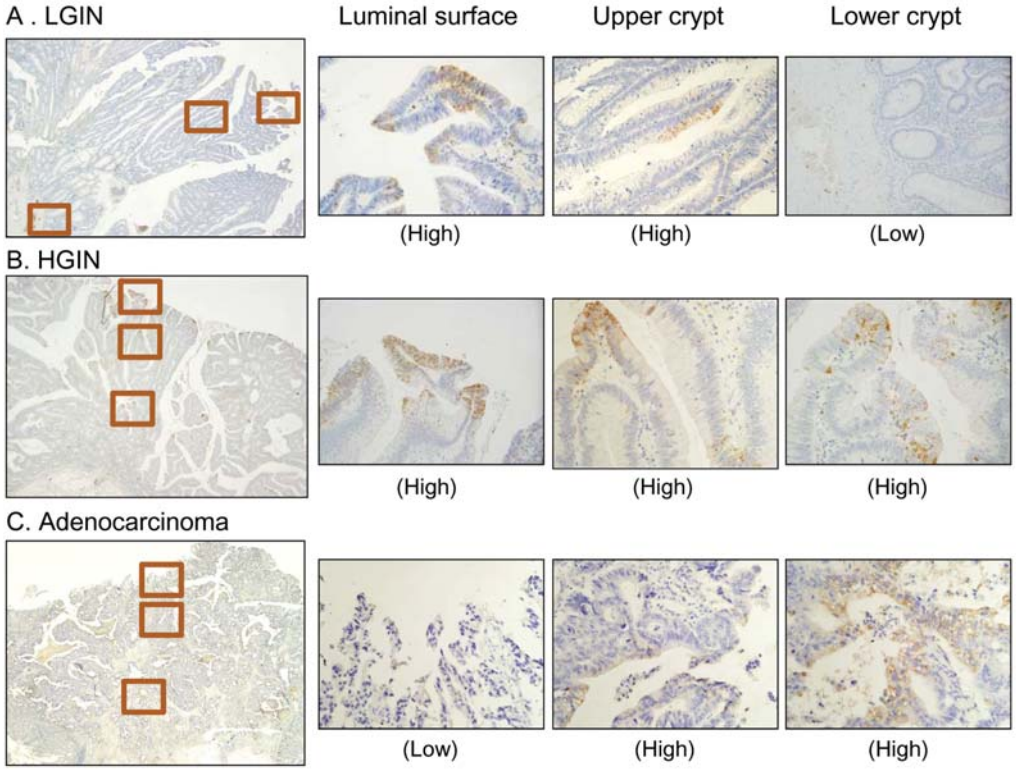

Figure 2. *Representative distribution patterns of LGR5 expression in tumors. A: LGIN, B: HGIN and C: adenocarcinoma (×28.8). High-power view of representative area in each tumor is shown in a box; LGIN: luminal surface (×400), upper crypt (×400) and lower crypt (×400); HGIN: luminal surface (×200), upper crypt (×400) and lower crypt (×400); adenocarcinoma: luminal surface (×400), upper crypt (×400) and lower crypt (×400). Each tumor area of the luminal surface, upper crypt or lower crypt was divided into a high and a low LGR5 expression group with cutoff level of 5% for the rate of LGR5-positive cells.*

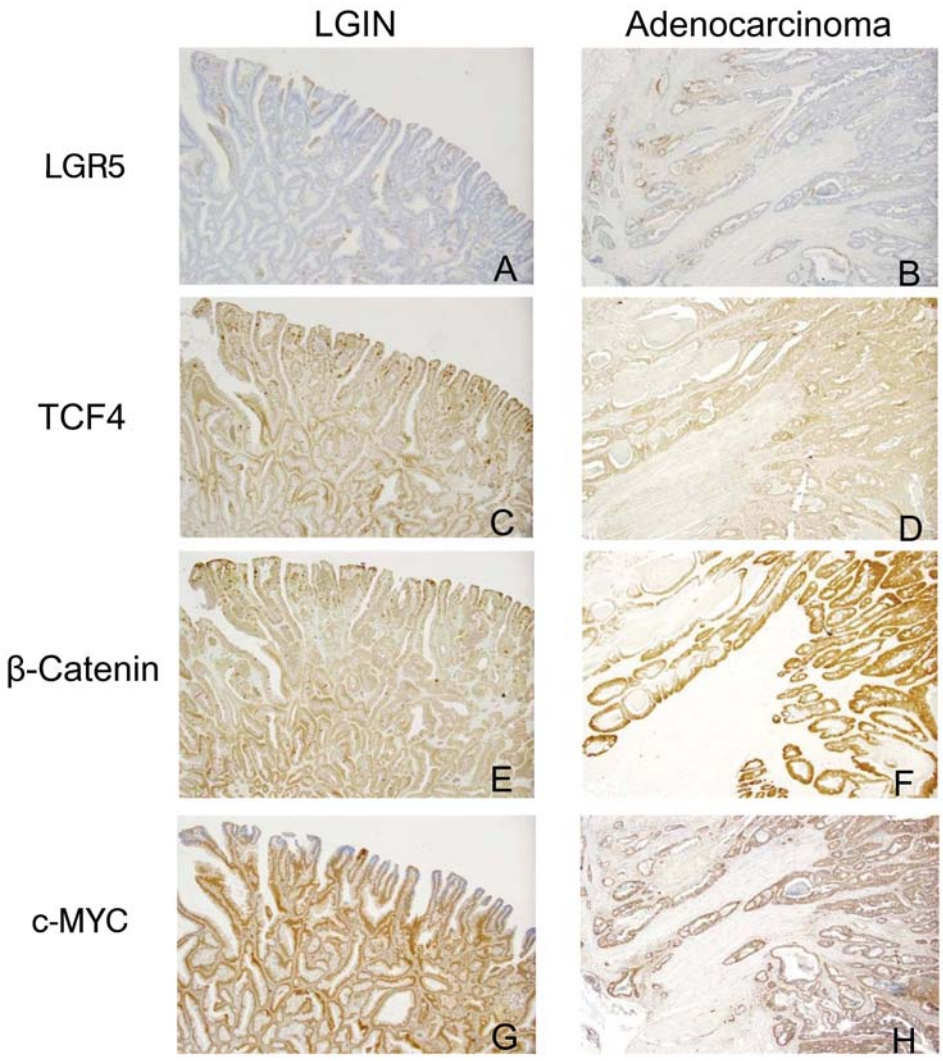

Figure 3. *Representative distribution patterns of expression of LGR5 and Wnt signaling proteins. Distribution patterns for LGR5 (A, B), TCF4 (C, D), β-catenin (E, F), and c-MYC (G, H) in LGIN (A, C, E, G) and adenocarcinoma (B, D, F, H) (×40).*

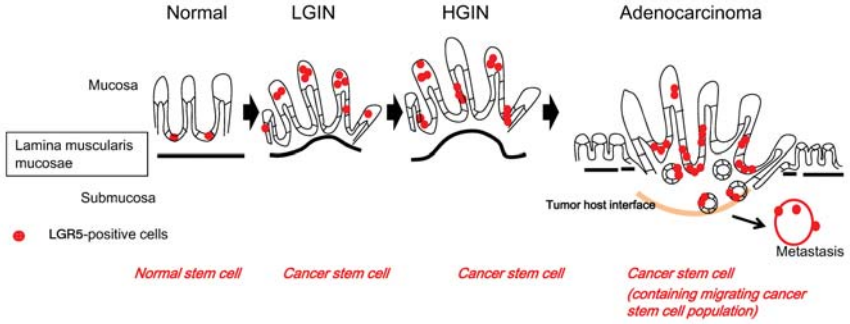

Figure 4. *A schematic model of distributional changes of LGR5-positive cells during each stage of colorectal tumorigenesis. LGR5-positive cells are located at the base of crypts in normal colorectal epithelia. Increased and clustered LGR5-positive cells are located at the luminal surface in LGINs, consistent with previously proposed top-down model, while they shift towards the crypt bottom and/or invasive tumor front in HGINs and especially in adenocarcinomas. The LGR5-positive cells are plausible cancer stem cells in human colorectal tumors, and may partly represent the migrating cancer stem cells in adenocarcinomas.*

Table II. *Relationship between distribution of LGR5 and clinicopathological characteristics in 60 resected human colorectal tumors.*

Characteristic		LGR5 expression								
		Luminal surface			Upper crypt			Lower crypt		
		Low	High	*p*-Value*	Low	High	*p*-Value*	Low	High	*p*-Value*
Gender	Male	15	17		13	19		14	18	
	Female	18	10	0.2	16	12	0.2	16	12	0.3
Tumor grade	LGIN	6	11		11	6		17	0	
	HGIN	3	10		6	7		6	7	
	Adenocarcinoma	24	6	0.0004	12	18	0.3	7	23	0.0001
Adenocarcinoma										
pT	T1-2	7	4		3	8		4	7	
	T3-4	17	2	0.2	9	10	0.5	3	16	0.4
pN	N0	12	6		7	11		5	13	
	N1-3	12	0	0.06	5	7	1.0	2	10	0.7
pStage	Stage I-II	11	6		6	11		5	12	
	Stage III-IV	13	0	0.02	6	7	0.7	2	11	0.4

*χ^2 test or Fisher's exact probability test as appropriate.

distributions of putative stem cells with LGR5 expression may shift from the luminal surface to the crypt base during the development of colorectal cancer. This study also showed that there was a negative association between LGR5 expression and pStage in the luminal surface of adenocarcinoma along with an invariably high expression of LGR5 in the lower crypt in advanced pStage disease. These findings suggest that a shift in the distribution of LGR5 expression towards crypt bottom and/or invasive tumor front may play an important role in the progression of colorectal cancer (Figure 4).

Although adenomas have been conventionally thought to develop from stem cells located at the base of normal crypts, this study found that the main location of the putative stem cells that expressed LGR5 was at the luminal surface in adenoma. This was especially the case in LGINs in the present study. These results are consistent with a previous study that reported a few cases of adenomas in which the LGR5-positive cells were commonly found at the luminal surface but rarely at the crypt base (18). Two models that have been proposed for adenoma morphogenesis. The Vogelstein group demonstrated that dysplastic cells at the tops of the crypts often exhibited genetic alterations of APC in early non-familial adenomatous polyposis (non-FAP) adenomas, while those cells located at the base of these same crypts did not contain such alterations (33). Based on these findings, they proposed a top-down model in which genetically altered cells in the superficial portions of the mucosae spread laterally and downward in order to displace the normal epithelium of the adjacent crypts (33). In contrast, Preston *et al*. analyzed the earliest lesions of sporadic and FAP adenomas and proposed a bottom-up model in which both sporadic and FAP adenomas start as monocryptal adenomas and then initially grow by crypt fission (34). However, they also found that in slightly larger sporadic adenomas, there was evidence of growth down into the adjacent crypts through the surface (34), which is consistent with the previously proposed top-down model. Both models imply the location of the mutant stem cells that are driving adenomatous growth. The findings of the present study are consistent with the top-down model, which suggests that stem cells might often be present in the luminal surface and upper portion of the mucosae in relatively large sporadic adenomas (Figure 4).

Table III. *Relationship between LGR5 expression and TCF4, β-catenin and c-MYC in 60 resected human colorectal tumors.*

Score		LGR5 staining rate		
		Low	High	*p*-Value*
TCF4†	Low	14	16	
	High	8	22	0.1
β-Catenin†	Low	13	16	
	High	9	22	0.2
c-MYC†	Low	10	20	
	High	12	18	0.6

*χ^2 test. †IHC score=(percentage of positive cells [percentage score])×(staining intensity [scored as 0 to 3]).

The shift in the distribution of the LGR5-positive cells towards the crypt base and/or invasive tumor front during the development and progression of colorectal cancer may be related to the migrating or mobile cancer stem (MCS) cells (Figure 4), as has been proposed by Brabletz *et al*. (35). To explain the heterogeneous morphology of the primary tumor and clarify how these metastases can recapitulate the heterogeneity, this group analyzed the expressions of a stem cell marker, survivin, and epithelial–mesenchymal transition (EMT) markers such as L1CAM in colorectal cancer progression. Based on their findings, they proposed the presence of two forms of cancer stem cells during the tumor progression: stationary cancer stem (SCS) cells and MCS cells. While SCS cells are already active in adenomas and persist in differentiated areas throughout all the steps of tumor progression, they are not able to disseminate. In contrast, MSC cells, which are derived from SCS cells that acquire a transient EMT, are located predominantly at the tumor-host interface or invasive tumor front and mediate tumor cell metastasis. In human pancreatic cancer, $CD133^+$ cancer stem cells are exclusively tumorigenic. However, only a distinct subpopulation of $CD133^+$ $CXCR4^+$ cancer stem cells within the invasive front are essential for tumor metastasis (36), which supports the MCS cell hypothesis. Taken together, the putative stem cells with LGR5 expression in the crypt base and/or invasive tumor front may partly represent the MCS cells, which may be essential for dissemination and metastasis.

LGR5, which was suppressed by dominant negative TCF4 in colorectal cancer cells, has been identified as a Wnt target gene (7, 37). Recent studies demonstrated that there was LGR5 overexpression in human hepatocellular carcinomas with β-catenin mutations (23) and that there was a correlation between the expressions of LGR5 and nuclear β-Catenin in human colorectal cancer (20). While this study found that there was no significant association between LGR5 and β-catenin or TCF4, there was a slight trend towards a positive relationship between them. However, the current results are more consistent with other findings that have shown that the deletion of *Apc* in the Lgr5 stem cells of *Lgr5*-EGFP-IRES-creERT2/$APC^{flox/flox}$ mice leads to their transformation (8). In these animals, the expression of Lgr5 was maintained in the transformed stem cells 36 days after the *Apc* deletion, only to be subsequently lost in most of the transformed cells, including the transient amplifying cells. In contrast, these cells were able to retain the high nuclear β-catenin and c-MYC expressions (8). Interestingly, LGR5-positive cells comprised 6.5% of the tumor population in that particular mice model (8), which was similar to the median expression rate of 5% that was observed in the present human colorectal cancer study. The broad expression of nuclear β-catenin and the other Wnt target, c-MYC, in the LGR5-negative cells may suggest the presence of pathways other than the Wnt pathway that are involved in LGR5 expression. This may possibly include the Hedgehog pathway that has been shown to be involved in the regulation of LGR5 in basal cell carcinomas (38).

In conclusion, the present study suggests that increases in putative stem cells with Lgr5 expression occur early during colorectal tumorigenesis and that shifts in their distribution towards the crypt bottom and/or invasive tumor front might play a role in the development and progression of colorectal cancer (Figure 4). Elucidation of LGR5 functions and the mechanism of its regulation may provide better understanding of colorectal tumorigenesis and may ultimately lead to the development of novel preventive and therapeutic strategies against colorectal cancer.

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